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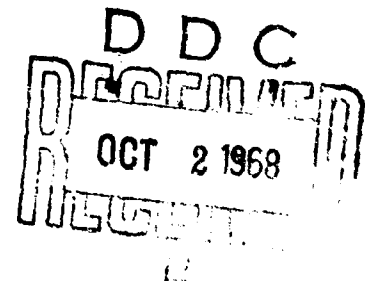
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STUDIES ON SUSCEPTIBILITY OF OCULAR TISSUES TO COWPOX VIRUS

Nihon Ganka Kiyo (Folia Ophthalmology Japan)
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I INTRODUCTION

It has previously been reported that herpes simplex and the pox virus can cause infections in the corneal epithelium and extensive research has been conducted on the cause and extent of virus infections in corneal epithelium [1-7].

However, despite the fact that opacity of the corneal cytoplasm and particles in the anterior chamber resulting from virus-caused corneal inflammations have been clinically examined, since the detection of inclusion bodies is extremely difficult, the presence or absence of infection in the cytoplasm or cells of the iris has not been clearly defined. Furthermore, in order to touch upon virus infections occurring in lens epithelium said to be completely free of inflammations or tumors and problems relating to whether they are the cause of white cataracts and whether they are the unknown cause of retinal inflammation, I have studied the susceptibility of various eye tissues (cornea, iris, lens, retina) of domestic rabbits to cowpox virus.

In cells infected by cowpox virus, two types of inclusion bodies, A and B, are developed [8,9]. The fact that the B-type inclusion bodies have a composition consisting of virus DNA [10] and the limiting part of the virus NP antigen [11] was shown by means of autoradiography using H^3 -thymidin as well as by the fluorescent antibody method. Furthermore, the A-type inclusion bodies are a continuous phenomenon in the formation of the B-type body and can be identified as a ring-shaped fluorescence [11] when dyed with anti-cowpox antigen complex fluorescent dye. Accordingly, both H^3 -thymidin autoradiography and the fluorescent antibody technique were used in this study as indices for cowpox virus growth.

The results are reported below.

II MATERIAL AND METHOD

1) Virus

Successive generations of cowpox virus (LB red strain) [12] and vaccinia virus (Biken strain) grown in an FL cell culture system were used as suspended solutions. Titters were approximately 10^6 pock forming units per ml in all cases.

2) Experimental Animals

Domestic white rabbits weighing approximately two kg were used.

3) Specimen Preparation and Staining Technique

Specimens for the fluorescent antibody method were prepared as follows after the removal of the eyeball:

- 1- After the cornea had been frozen with isopentane dry ice acetone, 7μ horizontal and vertical slices were taken from the corneal surface. Smear samples of corneal epithelium and endothelium were also made.
- 2- Endothelial smear samples as well as frozen sections of iris were made.
- 3- After immersing the lens in 0°C acetone for 5 seconds, a cross-incision was made at the extreme back and with a spatula, lens capsule as well as expanded samples of epithelial cells were made on a slide.
- 4- The retina was peeled off like a grape peel with a spatula and slide mounted 10μ slices were made.

The samples prepared in this manner were fixed with cold acetone and 10 minutes later were stained in a moist chamber for one hour at 37°C in a solution of fluorescein isothiocyanate combined with anti-cowpox virus rabbit γ -globulin. These were then washed thoroughly with a phosphoric acid buffer solution and mounted with glycerin. Examinations were then made with a Nikon S fluorescent microscope.

The fluorescent antibody solution was prepared according to Pettit[3].

In order to examine the inclusion bodies in the above-mentioned samples, hematoxylin-eosin (H.E.) stains were made following Bouin fixation. Also the samples examined by means of the fluorescent microscope were restained with H.E. after Bouin fixation and were examined with an optical microscope to observe the relationship between the parts giving rise to specific fluorescence with respect to the cell as well as the inclusion bodies.

For autoradiography, $10\mu\text{Ci}/0.1\text{ ml}$ of ^3H -thymidin (specific activity $4.32\text{Ci}/\text{mM}$, Amersham Biochemical Center, England) were injected into the anterior chamber and the vitreous body. After two hours the eyeball was

extracted, the cornea and retina were fixed with Cornoy solution and 5 μ paraffin sections were cut. Spread samples of lens epithelial cells were obtained according to the method of Harding [13]. After these samples were treated with 2% perchloric acid, autoradiography was carried out using NR-MI (Sakura) liquid emulsion with the dipping technique. After 2-6 weeks of exposure and developing in Konidol X for five minutes at 18°C, [samples] were stained with Giemsa solution and then examined [14].

4) Experimental Procedure

Inoculations of 0.05-0.1 ml of virus suspensions were made by scratching, as well as by injection into the cornea, by injection into the anterior chamber for the iris and lens and by injection into the vitreous body for the retina.

The cornea was scratched lightly in the form of a double cross and inoculated. In inoculating the lens, the hypodermic needle was inserted into the anterior chamber from the corneal rim and the lens was gently pierced with the needle point; then the virus suspension was introduced into the anterior chamber. The eyeball was removed at one, three and five days following virus inoculation and examinations were made with the three methods mentioned above.

III EXPERIMENTAL RESULTS

1) Clinical Findings

One day following inoculation of the corneal scratch, a slight opacity of the corneal cytoplasm appeared and examination using a slit lamp revealed primarily edema of the corneal surface layers as well as particles in the anterior chamber. This was followed by an advancing condition of the pox virus vaccinal keratitis. On the third day, eyelid edema, eye discharge, an affected conjunctiva as well as hair-like congestion became conspicuous. The cornea as well as virtually the entire stromal layers showed symptoms of edema and became greatly turbid.

Comparing the intra-corneal injection to inoculation on the scratch, the conjunctiva infection tended to be weaker.

With regard to the injection of the virus into the anterior chamber, after one day the anterior chamber particles, the resting particles at the back surface of the cornea and the iris congestion appeared strong and the cornea also was slightly opaque.

A day after the inoculation of the lens, light turbidity appeared centered at the wound.

In the inoculation of the vitreous body, after about three days edema as well as congestion of the conjunctiva, minute particles in the anterior chamber, and resting particles at the back surface of the cornea were noted.

After five days, symptoms of inflammation of the entire eye were evident.

2) Histological and Bacteriological Impressions

A) Cornea

a) Corneal Epithelial Cells (Diagram 1)

From the fluorescent antibody method, a light diffuse characteristic fluorescence was seen as if surrounding the nucleus (Figures 1, 2). With the cowpox virus, in addition to the faint fluorescence, a strong, ring shaped specific fluorescence was noted (Figure 3). Comparing impressions from corneal epithelial cells which were restained, that is, Bouin fixed and H.E. stained or methanol fixed and Giemsa stained, the light diffuse fluorescence corresponds to B-type inclusion bodies and the deep, ring-shaped fluorescence to A-type inclusion bodies. With the smear samples, most of the cells containing these types of inclusion bodies appeared clumped and also from the cut sections, in the infected part of the epithelium the infection penetrated all the layers from the surface to the basal cells. Occasionally, the inclusion bodies were found in the stromal cells immediately below the epithelium.

b) Corneal Stromal Cells (Diagrams 2,3,4)

From the fluorescent antibody method, in the cytoplasm of the stromal cells, considerable ring-shaped fluorescence and surrounding irregular specific fluorescence was seen (Figures 4, 5).

Upon examining sagittal sections, in the case of the inoculation by scratch which affected the stromal cells superficially, with the scratch as the center, several to several tens of cells were seen to demonstrate specific fluorescence in the epithelium and stromal cells (Figure 4). Even in the sections of the epithelium which were not infected, large numbers of infected cells were seen in the stromal cells running along the lamella. However, the infection only spread a little distance in depth from the wound. When the virus suspension was injected into the stroma, it spread along the lamella and formed circular plates. But in this case the virus infected cells spread sideways along the lamella and formed a focus of infection. Although the spread along the lamella is some distance from the infected cell grouping, specific fluorescence was seen.

From autoradiography, grains were not usually seen in the nuclei nor in the cytoplasm of nearly all the corneal stromal cells; however, on rare occasions, grains were seen in the nuclei of stromal cells. With extreme rarity, grains were seen in the limiting position of the cytoplasm of the corneal stromal cells (Figure 7).

c) Corneal Endothelial Cells (Diagram 5)

In the fluorescent antibody technique, numerous specific ring-shaped fluorescence and diffuse fluorescence surrounding them were seen in the cytoplasm of the endothelial cells from the smear samples (Figure 9).

A- and B-type inclusion bodies were identified in the Bouin fixed, H.E. restained [samples]. In the frozen sections, specific fluorescence in the endothelium was seen.

B) Iris (Diagrams 6 and 7)

In the fluorescent antibody method with smear samples of iris endothelium, diffuse specific fluorescence and intense ring-shaped fluorescence were seen in the cytoplasm (Figure 10). Following Bouin fixation and restaining with H.E., the ring-shaped fluorescence was seen to correspond to A-type inclusion bodies (Figure 11). Fluorescence was not noted in the cytoplasm from iris frozen sections; specific fluorescence was seen in the epithelial and endothelial layers (Figure 12).

C) Crystalline Lens (Diagram 8)

In the fluorescent antibody method on spread samples of lens epithelium, examination of specific fluorescence was somewhat difficult; however, diffuse and light specific fluorescence as well as numerous ring-shaped fluorescence was seen in the cytoplasm (Figure 13).

With Bouin fixation and H.E. staining, the reddish ring with halo A-type and brownish B-type inclusion bodies were identified (Figure 14).

In the autoradiography, grains corresponding to B-type inclusion bodies were noted in the cell cytoplasm. However, in those nuclei, grains were not seen (Figure 15). Moreover, at the present time, there is no evidence for virus infection of the lens epithelium in the absence of a wound on the capsule.

D) Retina (Diagrams 9, 10)

From the fluorescent antibody method, granular diffuse specific fluorescence and concentrated ring fluorescence in the cowpox virus infected cytoplasm were seen in all layers of the brain layers (inner granular to inner layers) (Figure 16). With Bouin fixation and H.E. stain, the A-type inclusion bodies which stain strongly with eosin were seen.

With autoradiography, finite grains corresponding to B-type inclusion bodies were seen in the cytoplasm of the ganglion cell layer and in the inner granular layer (Figures 17; 18). Although grains were not identifiable in the nuclei of the infected cells, grains were seen in parts of the ganglion cell nuclei.

IV DISCUSSION

Heretofore, evidence of virus infection of cells were given by identification of inclusion bodies by fluorescent microscopy or observation of virus particles by means of electron microscopy; however, in recent years, the use of fluorescent antibody technique and autoradiography have simplified identification. Also, with regard to infectious symptoms and intracellular nucleic acid metabolism, considerable new information has been

uncovered. Furthermore, from a clinical standpoint, rapid and specific diagnoses of corneal epithelial virus infections have become possible using the fluorescent antibody method and this is gradually coming into everyday practice [14-19].

I have used the fluorescent antibody method primarily and have noted the cowpox virus infection in cell tissues of the cornea (epithelium, cytoplasm, endothelium); iris (endothelium, epithelium); crystalline lens epithelium and the retina. Among these, the virus infection of the lens epithelium deserves most attention.

It has been said that the lens has never had inflammation or tumor (reports on virus infection of the lens epithelial cells have never appeared previously).

By using the fluorescent antibody method, autoradiography, and examination of the inclusion bodies, I have confirmed that cowpox virus does infect the epithelial cells of the lens. This is the first [reported] evidence of lens epithelial cell infection. This fact is a substantiation of the soundness of the thought that one of the causes of congenital white cataract is a virus infection by the German measles virus, for example, and probably the virus infection occurs prior to the formation of the lens capsule during gestation. Moreover, although virus infection was not seen in normal lens epithelial cells in the absence of a wound, when a change occurs for whatever reason, on the capsule, the virus particles pass through the capsule (virus particles 80-200m μ do not usually traverse the capsule) and the lens epithelial cells can become infected. Thus, coupled with the change in capsule permeability, it can also be thought that it is the cause of white cataracts [21].

The next interesting point was the separation of viral DNA from the nucleus DNA seen in the retinal cells infected with cowpox virus. The retina is differentiated in the early stages and is considered to be unable to regenerate. In my experiments, DNA synthesizing cells were not detected in the normal retina and in the retina of the virus inoculated eyeball, DNA synthesizing cells were only rarely detected in the ganglion cell layer. However, in concert with the ganglion cell layer as well as with nuclear inclusion bodies of the cells in the bipolar cell layer, considerable viral DNA synthesis was seen. This is thought to imply that even though the cell is unable to synthesize DNA, when DNA virus infection occurs, virus DNA synthesis arises.

Although there have been reports on retinal inflammation due to cytomegalic inclusion disease [21, 22], clinically, the causes of most retinal inflammation and chorioretinopathy remain unknown.

While I have shown that cowpox virus does infect the retina, Thomas, Uchida, et. al., at the same time, have reported the infection of the retina with herpes simplex virus. Thus it is probably necessary to consider virus

infection as a cause of retinal inflammation.

With regard to viral inflammation of the cornea, although there has been no corroboration that corneal stromal cells could be infected by virus in laboratory experiments with inclusion bodies or virus separated from tissue cultures, my experiments have clearly shown that cowpox virus infects corneal stromal cells. Although interesting results have been obtained relating to corneal cytoplasm opacity and virus infection, the details will be reported in the future.

V CONCLUSION

Cowpox virus was inoculated into eyeballs of domestic rabbits, inclusion bodies were noted in the cell cytoplasm of the cornea (epithelium, cytoplasm, endothelium); iris (epithelium, endothelium); lens epithelium and the retina (brain layer). These tissues were shown to be infected with the cowpox virus by means of the fluorescent antibody method and autoradiography.

In conclusion, deepfelt appreciation is extended to Professor Mizukawa for guidance and for his revisions. Also, the helping hand of Professor S. Kato was greatly appreciated.

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TRANSLATOR'S NOTE

- 1) Nichi Gan is probably the abbreviation for Nihon Ganka Gakkai Zasshi (Acta Societatis Ophthalmologica Japonicae).

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FIGURE APPENDIX

Fig. 2

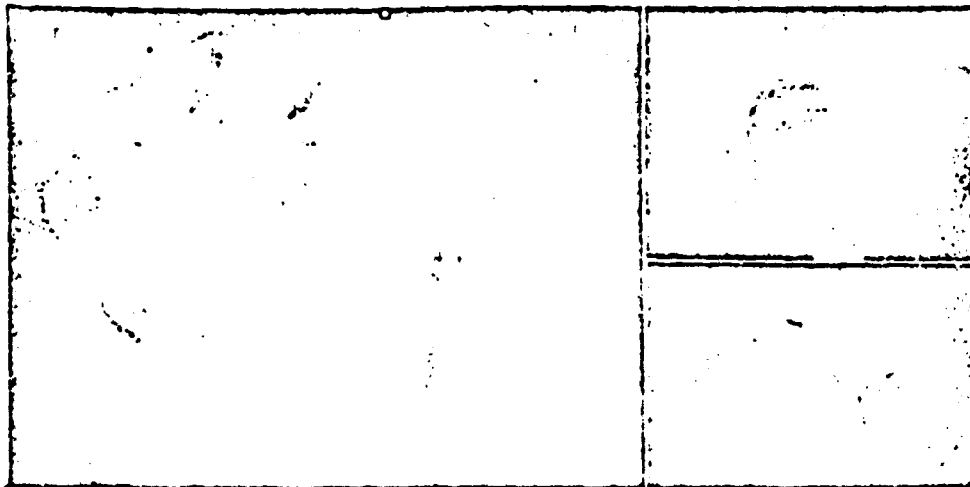


Fig. 1

Fig. 3

Fig. 1 Fluorescent antibody staining of smear preparation of rabbit corneal epithelium infected with variola Virus Biken strain (1 day after virus inoculation). Note specific fluorescences near the nucleus.

Fig. 2 Higher magnification of Fig. 1.

Fig. 3 Fluorescent antibody staining of smear preparation of corneal epithelium infected with cowpox virus. Note ring-shape fluorescences and irregular fluorescences around them in the cytoplasm correspond to "A" type and "B" type inclusions, respectively.

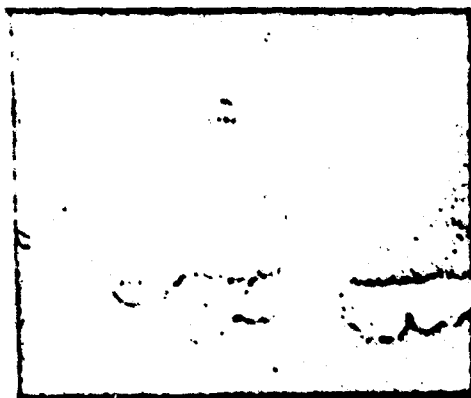


Fig. 4

Fig. 4

Fluorescent antibody staining of fresh frozen sagittal section of rabbit cornea infected with cowpox virus.

Intensive fluorescence in the cytoplasm of stromal cells and epithelial cells.

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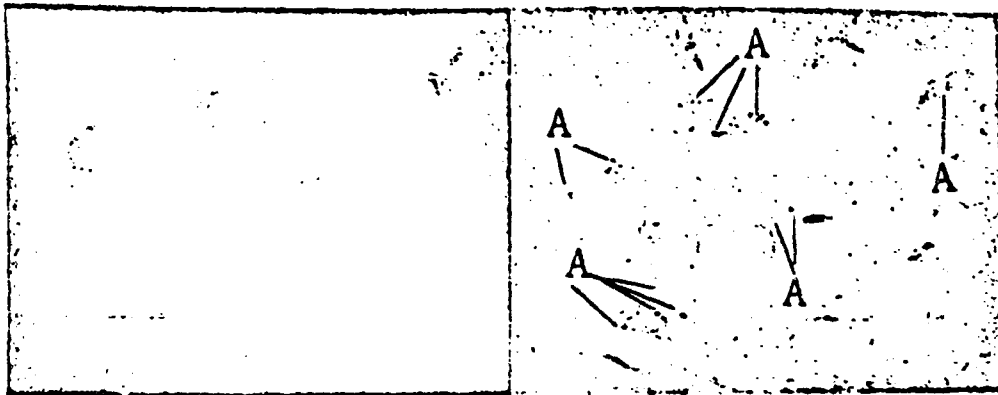


Fig. 5

Fig. 6

Fig. 5 Fluorescent antibody staining of fresh tangential frozen section of rabbit cornea infected with cowpox virus. (5 days after virus inoculation)
Intensive ring-shape fluorescence in the cytoplasm of stromal cells correspond to outline of the "A" type inclusions. (10 \times 40)

Fig. 6 Hematoxylin-eosin staining of the same field of Fig. 5
Intracytoplasmic "A" type inclusions of the stromal cell. "B" type inclusion is diffuse and obscure in this figure.
(10 \times 100, Bouin's fixation)
N : Nucleus of stromal cell,
A : "A" type inclusion

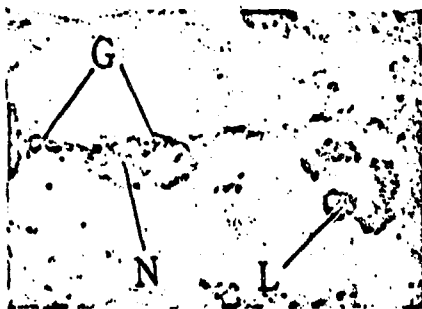


Fig. 7

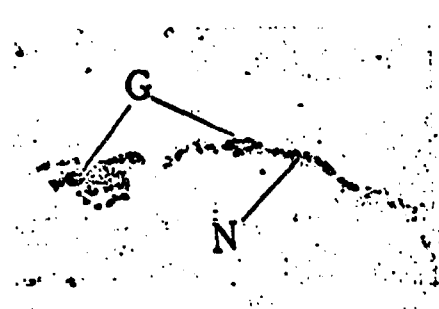


Fig. 8

Fig. 7 Fig. 8 Autoradiograms of paraffin section of rabbit cornea infected with cowpox virus. (1 day after virus inoculation) Silver grains are found in the cytoplasm of stromal cell, but not on the nucleus: These figures indicate that the intracytoplasmic viral DNA is synthesized without nuclear DNA synthesis in the infected stromal cell.

N : Nucleus of Stromal cell
G : Grain, L : Leucocyte.



Fig. 9

Fig. 9 Fluorescent antibody staining of smear preparation of corneal endothelium infected with cowpox virus (2 days after virus inoculation). Note ring-shape fluorescences correspond to "A" type inclusions.



Fig. 10

Fig. 10 Smear preparation of the endothelium of the iris infected with cowpox virus. Fluorescent antibody staining showing ring-shape and diffuse fluorescences in the cytoplasm.

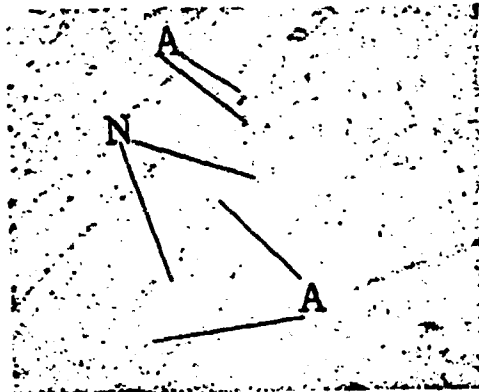


Fig. 11

Fig. 11 Hematoxylin-eosin restaining of the same field of Fig. 10. Note correspondence of ring-shape fluorescences to "A" type inclusion.



Fig. 12

Fig. 12 Fluorescent antibody staining of sagittal section of the iris infected with cowpox virus. Showing specific fluorescences in the endothelium and epithelium.

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Fig. 13



Fig. 14

Fig. 13 Fluorescent antibody staining of lens epithelium infected with cowpox virus (1 day after virus inoculation).
Showing fluorescence in the cytoplasm correspond to inclusions. (10×40)

Fig. 14 Hematoxylin-eosin staining of lens epithelium infected with cowpox virus showing "A" type inclusions and "B" type inclusions.
N : nucleus of lens epithelium,
A : "A" type inclusion, and
B : "B" type inclusion

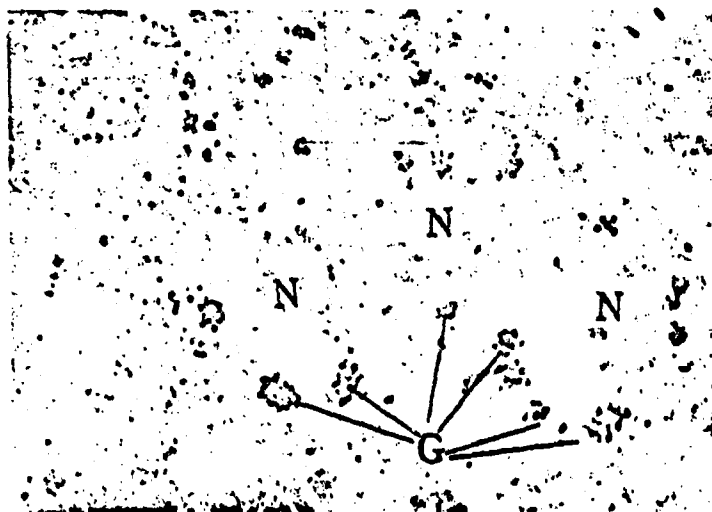


Fig. 15

Fig. 15 Autoradiogram of lens epithelium infected with cowpox virus (1 day after virus inoculation).
Note aggregation of silver grains in the cytoplasm corresponding "B" type inclusions of cowpox virus (10×100)

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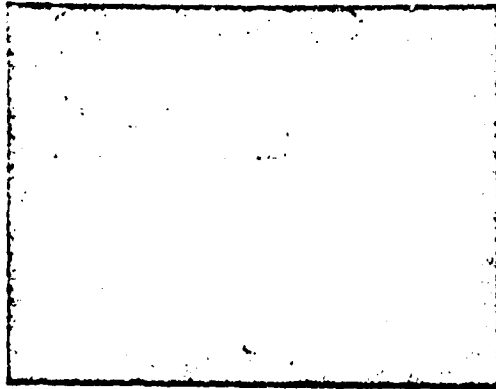


Fig. 16

Fig. 16 Fluorescent antibody staining of the retina infected with cowpox virus showing fluorescences in the inner layers from inner granular cell layer.



Fig. 17

Fig. 17 Autoradiogram of the retina infected with cowpox virus (5 days after virus inoculation). Note aggregations of silver grains in the cytoplasm of ganglion cells and bipolar cells. (10×40).



Fig. 18

Fig. 18 Higher magnification of Fig. 17. (10×100).